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Six-Month Exposure of Strain A/J Mice to Cigarette Sidestream Smoke: Cell Kinetics and Lung Tumor Data

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Male strain A/J mice were exposed to sidestream smoke (SS) generated from burning Kentucky 1R4F reference cigarettes. Chamber concentrations were 4 mg/m^3 of total suspended respirable particulate matter (TSP). Animals were exposed 6 hr a day, 5 days a week. One-week cumulative labeling indices were significantly increased in the large intrapulmonary airways during the 1st week and in the respiratory epithelium of the nasal and maxillar turbinates during the first 3 weeks of exposure and then returned to control values. Subsequently, signs of increased cell proliferation were again found in the nasal and maxillar turbinates during the 9th and 16th exposure weeks. The experiment was terminated after 6 months. The number of animals bearing lung tumors was the same in smoke-exposed as in filtered air-exposed animals as was the average number of tumors per lung. Analysis of the DNA of individual tumors obtained from exposed and control mice for K-ras mutations suggested that exon 2 might be a specific target for SS. It was concluded that (1) duration of exposure was too short or (2) concentration of TSP was too low to reveal a possible carcinogenic potential of SS in strain A/J mice or that (3) SS is not carcinogenic in strain A mice.

In 1992, the United States Environmental Protection Agency designated environmental tobacco smoke (ETS) as a known human or class A carcinogen (US EPA, 1992). ETS is a 85%–15% mixture of aged and diluted sidestream smoke (SS), the smoke curling off the end of a lit cigarette between puffs, and the mainstream smoke (MS) exhaled by active smokers. The class A categorization of ETS was based on a review of the available epidemiological evidence that described and analyzed adverse health effects in humans married to smokers. The overall evaluation included some 30 studies from different countries such as the United States, Japan, Greece, Western Europe, and Hong Kong. For the purpose of population risk assessment, an analysis of 11 epidemiological studies conducted in the United States was performed. It was concluded that in the United States approximately 3000 lung cancer deaths per year could be attributed to ETS (US EPA, 1992).

The conclusion that ETS is a human carcinogen was mostly drawn from the analysis of the epidemiologic data. It was reinforced by a consideration of the strength of evidence provided by other data. There is high biological plausibility that ETS is a carcinogen. The same carcinogenic chemicals that can be found in cigarette mainstream smoke are present in ETS (Guerin *et al.*, 1992). The carcinogenic potential of MS has been well documented in man and in experimental animals (IARC, 1986). However, no animal studies are available yet that might provide similar information on cigarette SS. The longest animal studies done with SS thus far lasted for only 90 days. In those studies, concentrations of SS in the inhaled air ranged from 1 to 10 mg/m^3 of total suspended particulates (TSP). No abnormal histopathological findings due to chronic SS exposure were found in the lungs. Signs of transient epithelial hyperplasia developed only in circumscribed locations within the nasal cavity of rats. Obviously, the two experiments were too short to produce a carcinogenic response in the two species that were examined, hamsters (von Meyerinck *et al.*, 1989) and rats (Coggins *et al.*, 1993).

We decided to examine the effects of exposure to SS in an experimental system where a positive response within 6 months could conceivably be found. Strain A/J mice respond readily with development of multiple lung tumors following exposure to many classes of carcinogens within 4 to 6 months after the beginning of exposure. Nitrosamines, carbamates or polycyclic aromatic hydrocarbons are highly tumorigenic in strain A/J mice (Shimkin and Stoner, 1975), whereas metallic compounds, organic drinking water contaminants, or aromatic amines elicit little or no response (Stoner *et al.*, 1976; Theiss *et al.*, 1977; Maronpot *et al.*, 1986). In practically all carcinogenicity tests done with strain A/J mice, the carcinogen was given by oral or intra-

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TABLE 8
Extent of Agreement between Assays

	BCO-P	CAMVA	Eytex	TOPKAT
BCO-P	—			
CAMVA	29/35 (83%)	—		
Eytex	28/35 (80%)	28/35 (80%)	—	
TOPKAT	9/15 (60%)	11/16 (69%)	10/15 (67%)	—

Note. The fraction given is the number of compounds for which the intersecting assays gave the same classification (i.e., nonirritating or irritating) per the number of compounds tested in both assays. The percentage agreement is given below this ratio.

tion. While the literature indicates that cytotoxicity assays can correlate well with *in vivo* eye irritation data when comparisons are made within a single chemical class (Borenfreund and Borrero, 1984; Shopsis and Sathe, 1984; Booman *et al.*, 1989), the cytotoxicity assays tested here showed little predictive value for this heterogeneous group of materials. These results underscore the fact that there should not be a single or limited number of assays or protocols adopted as an alternative(s) to the Draize eye test. Rather, the needs of a laboratory should be identified and then a method chosen for development. If the goal is to develop an assay which is robust across a variety of chemical classes, this study suggests that the BCOP assay would be the method of choice. However, there is insufficient reason to exclude the CAMVA and Eytex assays from further consideration based solely on a database of 37 compounds, particularly when each of these tests has been reported to be successful in other testing situations (Bagley *et al.*, 1988; Soto and Gordon, 1990; Regnier *et al.*, 1994).

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peritoneal administration. In only two studies were A/J mice exposed via the inhalation route for 6 months to a few known carcinogens such as vinyl chloride, bis(chloromethyl)ether, ethylene oxide, or urethan aerosol. In both studies an unequivocally positive response was found for these chemicals (Leong *et al.*, 1971; Adkins *et al.*, 1986). Since strain A/J mice appear to provide a sensitive and short-term bioassay, it was of interest to examine the effects of SS under defined conditions of exposure.

MATERIALS AND METHODS

Animals. Male strain A/J mice, 6 to 8 weeks old, were purchased from Jackson Laboratories (Bar Harbor, ME). Selection of one sex only was dictated by constraints in available chamber space. Upon arrival, randomly chosen animals were sent to the Comparative Pathology Laboratory (UC Davis) for a standard health screen; all tests were negative with the exception of microbiological evidence for the presence of *Pasteurella pneumotropica* in the nasopharynx (no significant gross lesions present). The animals were housed, four to a cage, in polypropylene boxes with tightly fitting wire screen lids on conventional bedding material. The animals had free access to conventional laboratory chow and to water *ad libitum* throughout the experiment, including when being exposed to smoke.

Materials. Reference cigarettes (Kentucky 1R4F) were purchased from the Tobacco Research Institute (University of Kentucky, Lexington, KY). Alzet Model 2001 osmotic minipumps with a nominal pumping rate of 1 μl/hr were purchased from Alza Corporation (Palo Alto, CA) and 5-bromo-2'deoxyuridine (BrdU) from Sigma Chemical Co. (St. Louis, MO). Anti-BrdU antibody was obtained from Boehringer-Mannheim (Indianapolis, IN) the Peroxidase Vecto Stain Elite ABC Kit, mouse IgG, from Vector Laboratories (Burlingame, CA), and Immuno-Bed from Polysciences, Inc. (Warrington, PA).

Experimental design. In the carcinogenesis study, a total of 96 mice were divided at random into two groups, group S and group A. The animals were placed into glass and stainless steel Hinners-type exposure chambers (volume, 0.44 m^3) when they were 13 to 15 weeks old. Group S (12 cages) was exposed for 6 hr a day, 5 days per week (Monday through Friday) to SS produced from burning Kentucky 1R4F reference cigarettes. The target concentration in the chamber was 4 mg TSP per m^3. The animals remained in their plastic cages within the inhalation chamber for the entire duration of the experiment. Bedding was changed once every week. In the chambers, the cages were placed on a top and a bottom rack, 6 cages on each rack in a front and back row. Every week, the position of all cages was rotated in a systematic way so that during the entire experiment each cage occupied at least twice each of the 12 possible positions within the chamber. Group A (12 cages) was kept in an adjacent room in a similar inhalation chamber ventilated with filtered air. The animals were observed daily and weighed weekly. The experiment was terminated after 6 months of exposure. Twelve animals from each group were assigned for detailed histopathological analysis of lungs and nasal passages; these findings will be reported elsewhere. The lungs of the remaining 36 animals in each group were processed for determination of tumor incidence and number.

In a parallel study, the cumulative labeling indices in the respiratory tract of SS- and of filtered air-exposed A/J mice were determined during weeks 1, 2, 3, 4, 6, 9, and 16 of the experiment. These animals were kept in a second inhalation chamber with a similar target concentration for TSP. Actual chamber concentrations are given in Table 1. At the beginning of each week during which cumulative labeling indices were to be measured, six animals exposed to SS and six animals kept in filtered air were lightly anesthetized with methoxyflurane and received an osmotic minipump filled with BrdU solution (20 mg/ml) implanted under the skin of the back.

TABLE 1
Average Exposure Data

Parameter	Chamber 1	Chamber 2
Relative humidity (% RH)	41.0 ± 12.0 (118)	47.5 ± 6.7 (74)
Temperature (°C)	24.8 ± 1.1 (118)	25.1 ± 1.1 (74)
Carbon monoxide (ppm)	17 ± 2 (118)	17 ± 2 (74)
Nicotine (μg/m^3)	1011 ± 289 (117)	1122 ± 207 (69)
Total suspended particulate matter TSP (mg/m^3)	4.1 ± 0.4 (117)	4.5 ± 0.6 (71)

Note. Average exposure conditions in the two SS chambers. Chamber 1 housed the animals for the 6-month study, whereas the animals used in the cell kinetic studies were housed in chamber 2. All values are means ± SD from average daily measurements; the number of measurements is given in parentheses. TSP data represent measurements by weighing material collected on filters. In the control rooms, average relative humidity was 51.9 ± 10.4% and average temperature 22.6 ± 0.7°C; CO was never found to be higher than 1 ppm.

One week after implantation of the minipump, the animals were killed by pentobarbital overdose and the lungs processed for immunohistochemistry (described below).

Exposure system. The SS exposure system was identical to the one described by Teague *et al.* (1994) and used by us in previous studies (Witschi *et al.*, 1994; Rajini *et al.*, 1994a; Rajini and Witschi, 1994). Briefly, SS was generated by burning Kentucky 1R4F reference cigarettes in a smoking machine. The cigarettes were stored at 4°C until needed and at least 48 hr prior to use were placed in a closed chamber at 23°C along with a mixture of glycerin/water to establish a relative humidity of 60%. The cigarettes were smoked with standardized 35-ml puffs of 2 sec duration, once every minute, for a total of eight puffs per cigarette. The SS given off the tip of the smoldering cigarette between puffs was drawn, after dilution and aging in a conditioning chamber (2 min), into the exposure chambers. The chamber atmosphere was monitored for CO, nicotine, and TSP.

Chamber concentrations of total suspended particulate matter were monitored every 30 min for 2 min in the conditioning chamber and within the exposure chamber by a piezobalance (TSI Instruments, St. Paul, MN) and a PDM-3 MiniRam forward light scattering device (MIE, Inc., Billerica MA), calibrated by gravimetric method (weight of particles collected on Teflon filters). Total suspended particulate matter was measured two to three times a day by weighing material collected on filters. Particle size distribution was measured using a Royco 236 Laser particle counter (HIAC/Royco Instruments, Menlo Park, CA). The mass median diameter was 0.26 μm with a geometric standard deviation of 1.41. TSP measurements taken at the breathing zone of the animals, immediately above the bedding in empty cages, gave the same value as did measurements on air concentrations taken at the sampling port used when the cages within the chamber contained animals.

Carbon monoxide was monitored every 30 min, on an average 12 times daily, with a Model 880 non-dispersive-infrared (NDIR) analyzer (Beckmann Industries, La Habra, CA). Nicotine concentrations in the exposure chamber were measured by drawing air samples once or twice a day through sorbent tubes. The tubes were extracted with HPLC-grade ethyl acetate containing 0.1% triethylamine and the extract was analyzed in a gas chromatograph (Varian 3740) equipped with a DB-5 30-m × 0.53-

mm column (film thickness 1.5 μm) and a nitrogen-selective thermionic specific detector. Temperature and relative humidity within the chambers were monitored with an appropriate probe located within the chamber (Rustrack, St. Paul, MN). Actual exposure data measured over the 6-month period are given in Table 1.

Tissue preparation. For analysis of tumor incidence and multiplicity, the lungs were inflated manually with Tellyesniczky's fluid and fixed for at least 24 hr. The number of tumor nodules visible on the lung surface was counted by two different observers. The procedure has been shown to accurately give the total number of all macroscopically discernible tumors in a mouse lung (Witschi, 1981). The results were expressed as tumor incidence, i.e., percentage of animals with one or several lung tumors and as tumor multiplicity, the average number of tumors per lung either per tumor bearing animal or per all animals, i.e., including animals without tumors.

For determination of labeling indices, the lungs were inflated through the trachea with 1% paraformaldehyde/0.1% glutaraldehyde solution. One hour later the lungs and the trachea were transferred into 70% ethanol, dehydrated in 95 and 100% ethanol, placed in Immuno-Bed (all lung lobes in two blocks and several cross sections of the trachea into another block), and cut on a Sorvall JB 4 microtome into 1- to 2-μm-thin sections. The sections were mounted on glass slides precoated with poly-L-lysine. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide in water, followed by an incubation with pronase (5.4 units/mg; 0.1 mg/ml for 30 min at 37°C) until the activity was stopped with undiluted calf serum. The sections were incubated with 2 N HCl for 30 min at 37°C, washed, treated with normal horse serum, washed, and incubated for 1 h at room temperature. They then were incubated with anti-BrdU antibody (1:50), biotinylated secondary antibody and finally with diaminobenzidine substrate at concentrations and times given in the manufacturer's instructions. Counterstaining was done according to Bennett *et al.* (1976) with methylene blue and basic fuchsin.

The nasal cavities were processed as described by Young (1981) and by Henderson *et al.* (1993). At autopsy, the nasal passages were flushed retrograde through the nasopharyngeal orifice with Carnoy's fixative (60% ethanol, 30% chloroform, 10% acetic acid, v/v). After at least 24 hr in fixative, the tissue was decalcified in 13% formic acid for approximately 1 week and rinsed thoroughly in tap water. The nasal cavity was sectioned transversely immediately posterior to the incisor teeth and 1–2 mm anterior to the incisive papilla. The tissue was embedded in paraffin and 5-μm sections were cut on a conventional microtome. After deparaffinizing, the sections were stained essentially as described for the lungs, except that endogenous peroxidase activity was blocked with 1% hydrogen peroxide in methanol.

Evaluation of cell labeling. All slides were coded and counted without knowing the treatment. Labeling indices were determined for the following regions in the respiratory tract: alveolar zone, terminal bronchioles, intrapulmonary large airways, trachea, and nasal passages. In the alveolar zone, 500 to 1000 cells per lung were counted in randomly selected fields and cells were differentiated into type II epithelial cells (identified by their cuboidal shape and location mostly in the corner of the alveoli) and cells in the alveolar wall (endothelial and interstitial cells; Witschi and Morse, 1983). In the conducting airways, all cells that form the pseudocolumnar epithelium were counted as in our previous studies (Rajini *et al.*, 1993) and no attempt was made to further differentiate between basal cells and other cell types. A minimum of 500 cells per lung was counted in each of the large and peripheral airways and trachea. Terminal bronchioles were identified by their opening into alveolar ducts and intrapulmonary large airways by their diameter (0.5 to 1.5 mm). In the nasal passages, 500 cells each were counted in the respiratory epithelium of the nasal septum, the nasal turbinates, and the maxillary turbinates. The labeling index was calculated as the percentage of labeled epithelial cells of the total number of epithelial cells counted. For determination of the labeling index in the olfactory epithelium, the region of the dorsal meatus was selected (Johnson *et al.*, 1990) and the labeling index was defined as number of labeled cells per unit length of basement membrane (Monticello *et al.*, 1990).

TABLE 2
Carcinogenesis Study: Body Weights during Exposure

Time (months)	SS exposed	Filtered air exposed
0 (Exposure beginning)	24.0 ± 1.8	24.0 ± 1.9
1	25.3 ± 1.8	24.7 ± 1.8
2	26.0 ± 2.0	25.7 ± 1.8
3	26.6 ± 1.8	26.0 ± 1.8
4	27.1 ± 1.8	27.1 ± 2.0
5	27.4 ± 2.1	27.3 ± 2.0
6 (Terminate)	28.0 ± 2.2	27.8 ± 1.9

Note. The table gives the body weights of the animals in the carcinogenicity portion of the experiment. At the beginning of the experiment, the animals were 11–13 weeks old. All data are given as mean ± SD. The number of animals in each group at every time point was $n = 48$.

Determination of K-ras mutation spectrum in tumor DNA. The DNA from 14 individual lung tumors from control and from 11 tumors from SS exposed animals was extracted by using proteinase K. Exons 1 and 2 of the K-ras gene were amplified as described previously (Oreffo *et al.*, 1993). The PCR was carried out with mouse-specific primers, and the annealing temperature for PCR was maintained at 50°C. Exon 1 was amplified using sense primer 5-CTTGTGTGAGACATGTTC-3 and antisense primer 5-CTCTATCGTAGGGTCGTACT-3 to generate a 164-bp product. Exon 2 was amplified using sense primer 5-ACTCCTACAGGAAACCAAGT-3 and antisense primer 5-CTATAATGGTGAATATCTTC-3 to generate a 179-bp PCR product. Single-stranded PCR products used for direct sequencing were generated from the double-stranded PCR product of a 40-cycle reaction using appropriate nested primers. The sense internal primer 5-TTATTGTAAGGCCTGCTGAAAATGACTGAG-3 was used to generate a 150-bp PCR product for exon 1. Exon 2 single-stranded sense product (152 base pairs) was generated using internal primer 5-GATGGAGAAACCTGTCTC-3, while the antisense product (133 bp) was generated using internal primer 5-ATACACAAAGAAAGCCCTCC-3. PCR products were cleaned using a Qiagen QIAquic Spin PCR purification kit prior to sequencing with USB sequencing kit (United States Biochemicals). The appropriate nested primer *not* used to generate single-stranded PCR product was used in the sequencing reaction.

Statistical analysis. All numerical data were calculated as mean and SE. Data on labeling indices were converted to logarithms for statistical analysis. Comparisons of labeling indices between SS-exposed and corresponding age-matched air-exposed controls at any given time point were analyzed by Student's *t* test. Tumor incidences were compared using Fisher's exact test. A P value of 0.05 or less was considered to be significant.

RESULTS

All animals survived the 6 months of exposure to SS or to filtered air. The mice gained weight during the experimental period. At no time point was there a significant difference in body weights between the SS exposed animals and the air controls. The data are presented in Table 2.

Seven-day cumulative labeling indices were measured weekly in several regions of the respiratory tract during the first month and during the 6th, 9th, and 16th weeks of exposure. In the respiratory epithelium lining the nasal and

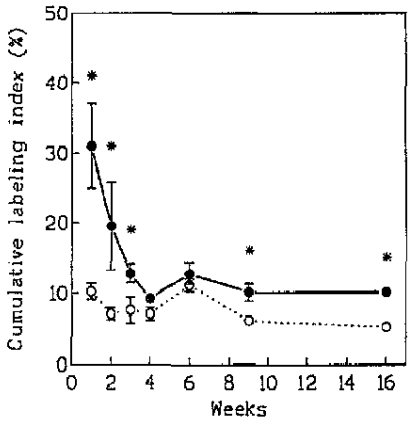

FIG. 1. Cumulative labeling indices (percentage of labeled cells) in the respiratory epithelium of the nasal turbinates. Experimental animals (filled circles) were exposed for 6 hr a day to sidestream smoke produced by burning Kentucky 1R4F reference cigarettes (chamber concentration 4 mg/m³ of TSP). Control animals (open circles) were kept in filtered air. Cumulative labeling indices were determined during the weeks indicated on the abscissa. All data are plotted as mean ± SE; where there is no SE plotted, it was smaller than the symbol. The number of animals per group was $n = 6$ throughout. Values labeled with an asterisk were significantly different ($P < 0.05$) from those of the corresponding control group.

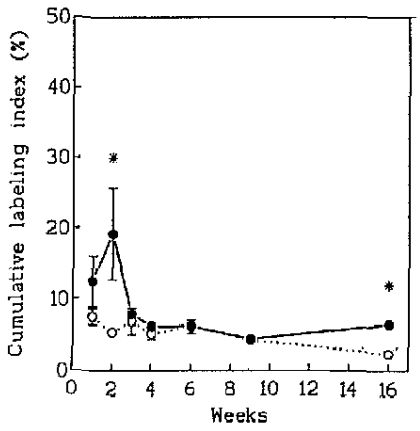

FIG. 3. Cumulative labeling indices (percentage of labeled cells) indices in the respiratory epithelium of the nasal septum. Animals were exposed to sidestream smoke (filled circles) or to filtered air (open circles). All conditions identical to the ones described for Fig. 1. Values labeled with an asterisk were significantly different ($P < 0.05$) from those of the corresponding control group.

maxillar turbinates, a significantly increased incorporation of BrdU was seen during the 1st 3 weeks (Figs. 1 and 2). Afterward, values in SS-exposed animals were close to control values, except at 9 and 16 weeks in the nasal and at 16 weeks in the maxillar turbinates. In the nasal septum, labeling indices were significantly higher only at 2 and 16 weeks (Fig. 3). Labeling indices measured in the olfactory epithelium that lines the dorsal meatus in the anterior nasal cavity were at all times higher or at least equal to control values; a statistically significant difference was seen only at 3 weeks (Fig. 4).

In the airways, responses to SS exposure were much less pronounced. A significantly higher labeling index in SS-exposed animals than in filtered air controls was found during the 1st week in the large airways (Table 3). The abnormally high control values seen during the third week in the airways was caused by very high values found in three animals that had been housed in the same cage. Labeling indices were also measured in the trachea and in the alveolar re-

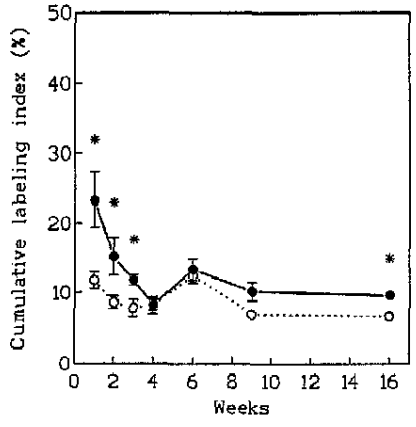

FIG. 2. Cumulative labeling (percentage of labeled cells) indices in the respiratory epithelium of the maxillar turbinates. Animals were exposed to sidestream smoke (filled circles) or to filtered air (open circles). All conditions identical to the ones described for Fig. 1. Values labeled with an asterisk were significantly different ($P < 0.05$) from those of the corresponding control group.

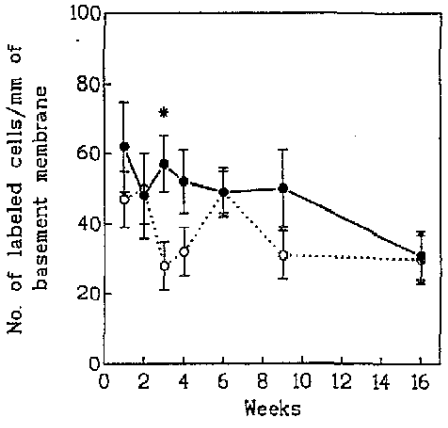

FIG. 4. Cumulative labeling indices (number of labeled cells per millimeter of basement membrane) in the dorsal meatus of the anterior nasal cavity in animals exposed to SS (filled circles) or to filtered air (open circles). All values represent the mean ± SE from six animals, except for SS-exposed at 2 and 16 weeks, where $n = 4$, and SS-exposed at 9 weeks, where $n = 5$. Values labeled with an asterisk were significantly different ($P < 0.05$) from those of the corresponding control group.

TABLE 3
Labeling Indices in Airways

	Large airways		Terminal bronchioles	
Week	SS	Air	SS	Air
1	8.0 ± 1.2[a]	4.4 ± 0.9	6.6 ± 1.4	3.5 ± 0.8
2	4.3 ± 0.6	3.9 ± 0.6	3.5 ± 0.7	4.5 ± 1.1
3	3.2 ± 0.7[a]	9.8 ± 2.0	4.6 ± 0.2[a]	7.4 ± 1.0
4	5.0 ± 0.7	2.9 ± 0.8	3.6 ± 0.6	2.9 ± 0.5
6	3.2 ± 0.9	4.2 ± 0.9	3.8 ± 0.6	3.8 ± 0.8
9	4.8 ± 0.4	3.3 ± 0.4	4.2 ± 0.3	4.5 ± 0.8
16	5.1 ± 1.2	4.4 ± 1.4	5.6 ± 1.3	2.7 ± 0.6

Note. All data are calculated as percentage of labeled cells per total number of cells counted and are given as means ± SE with $n = 6$ throughout, except for controls at 3 weeks, where $n = 3$ only.

[a] $P < 0.05$ compared to controls.

TABLE 5
Incidence and Multiplicity of Lung Tumors

Parameter	Smoke exposed	Air controls
Number of animals examined	36	36
Number of animals with lung tumors	12 (33%)	12 (33%)
Number of animals with one lung tumor	9	10
Number of animals with two lung tumors	3	2
Tumor multiplicity, all animals	0.42 (SD 0.65)	0.39 (SD 0.60)
Tumor multiplicity, tumor bearing animals only	1.25 (SD 0.45)	1.07 (SD 0.39)

Note. All animals were exposed for 6 hr a day, 5 days a week to SS at a nominal chamber concentration of 4 mg/m^3 of total suspended particulates. The number of tumors per lung was counted after 6 months' exposure.

gion. At no time was there a statistically significant difference between animals that had been exposed to SS and their controls (Table 4). The percentage of labeled type II cells in the lungs of smoke-exposed animals was never significantly different from control values (data not shown).

Data on lung tumors are presented in Table 5. The percentage of tumor-carrying animals was the same in the two groups and so was the average number of tumors per lung. A few lung tumors were examined under the light microscope. They showed the morphological features that are typical for lung tumors in mice. The tumors displayed a uniform adenomatous pattern with columns of cuboidal cells, uniform in size and shape, supported by a small amount of connective tissue without any capsule. The DNA from 11 individual tumors from SS-exposed animals and from 14 individual tumors from control animals was extracted and analyzed for K-ras mutations. The data are presented in Table 6. In SS-exposed mice, all lung tumors had K-ras mutations, whereas in control animals mutations were found in only 79% of the tumors. Tumors from SS-exposed animals had a different mutation spectrum: more than 90% had a mutation in exon 2 (codon 61), whereas in control animals mutations in exon 1 and exon 2 appeared to be equally distributed. The difference in K-ras mutation pattern between SS-exposed animals and controls was statistically significant.

TABLE 4
Labeling Indices in Trachea and Alveolar Zone

	Trachea		Alveolar zone	
Week	SS	Air	SS	Air
1	6.7 ± 1.5	6.0 ± 0.8	4.1 ± 0.5	4.2 ± 0.6
2	5.4 ± 0.6	5.5 ± 0.7	4.2 ± 0.5	4.2 ± 0.7
3	6.0 ± 1.1	5.9 ± 0.6	3.9 ± 0.3	3.9 ± 0.4
4	4.8 ± 0.7	3.0 ± 0.5	3.1 ± 0.3	2.9 ± 0.2
6	7.3 ± 1.1	7.1 ± 1.0	4.3 ± 0.4	5.4 ± 0.8
9	7.7 ± 1.3	5.9 ± 0.5	2.5 ± 0.4	4.2 ± 0.6
16	7.2 ± 1.2	8.9 ± 0.9	3.5 ± 0.5	3.2 ± 0.5

Note. All data are calculated as percentage of labeled cells per total number of cells counted and are given as means ± SE, with number of animals $n = 6$ per group throughout.

DISCUSSION

The main conclusion that must be drawn from the present experiment is that exposure to SS for 6 months and at a concentration of 4 mg/m^3 of TSP produces few effects in the lungs of strain A/J mice. Increased cell proliferation was mostly seen in the nasal passages and to a lesser degree in the large airways. Unequivocal changes were limited mostly to the first 3 weeks of exposure and then subsided, although some increased cell proliferation was again found after 3 to 4 months in the epithelium lining the nasal turbinates. At the end of 6 months, no increased incidence in lung tumors was found and the average number of tumors per lung was practically identical in SS-exposed and control animals.

The observations made on cell proliferation essentially agree with our findings made in earlier studies (Rajini and Witschi, 1994). As in the previous study, we could not find any changes in the pulmonary parenchyma. An initial increase in the labeling index was seen in the large intrapulmonary airways, although the response was not as pronounced as had been seen before. The complete disappearance of signs of increased cell proliferation seen after 1 to 2 weeks at this particular site is consistent with findings made in animals exposed to other irritating agents such as ozone where an initial increase in the labeling indices is usually

TABLE 6

Treatment	No. of tumors analyzed	No. of tumors with mutations	Tumors with mutations in exon 1 (codon 12)[a]	Tumors with mutations in exon 2 (codon 61)
A: K-ras mutation frequency in individual lung tumors[b]				
Air[c]	14	11 (79%)	5 (36%)	6 (43%)
SS[d]	11	11 (100%)	2 (18%)	10 (91%)[e]

B: Pattern of K-ras mutations in individual lung tumors[f]

	Codon 12				Codon 61			
	Normal	Mutations			Normal	Mutations		
	GGT Gly	GTT Val	GAT Asp	CGT Arg	CAA Gln	CGA Arg	CTA Leu	CAC His
Air[c]			4	1		3	2	1
SS[d]		1	1			7	3	

[a] No mutations were found in codon 13.
[b] DNA was extracted for analysis from individual tumors.
[c] Animals kept in filtered air.
[d] Animals exposed to SS for 6 months.
[e] Mutation frequency significantly higher ($P < 0.05$) when compared to mutation frequency in exon 2 in all tumors from air-exposed animals using Fisher's exact test with Yeates' correction.
[f] Sequencing analysis for the DNA from individual tumors.

followed by an attenuated response that often is no longer distinguishable from controls (Nikula *et al.*, 1988; Rajini *et al.*, 1994b; Rajini and Witschi, 1995).

Clear evidence for increased cell proliferation was found in the nasal passages. During the first 3 weeks, cumulative labeling indices were significantly higher in the respiratory epithelium that lines the nasal and maxillar turbinates. The response in the olfactory epithelium was less clear-cut. After the 3rd week, labeling indices in the exposed animals were close to those found in the filtered air controls. Toward the end of the observational period, the labeling indices in the exposed animals were again significantly higher than in the corresponding age-matched controls; the phenomenon was most pronounced for the nasal turbinates. It is interesting to note that this is the same location where Coggins *et al.* (1993) found slight to mild epithelial hyperplasia in rats exposed to 10 mg/m^3 of particulate matter. Similar changes were found in the same location in rats exposed to 4.3 mg/m^3 of particulate matter (Von Meyerinck *et al.*, 1989). In both studies the changes were reversible upon removal of the animals from smoke into air and may have represented adaptive rather than toxic changes (Burger *et al.*, 1989).

Analysis of lung tumor incidence and multiplicity failed to reveal any tumorigenic effect in SS-exposed animals. It must be understood that the negative data reported in this study do not imply that inhalation of ETS is "safe." There is both epidemiological evidence and biological plausibility that exposure to ETS constitutes a carcinogenic risk to people (US EPA, 1992; Pershagen, 1994). Rather, the experiment raises two questions: how sensitive a system is the strain A/J mouse lung tumor assay and how easy is it in general to produce lung tumors in mice with cigarette smoke.

In a strain A/J mouse lung tumor assay, usually two parameters are evaluated. One is tumor incidence, i.e., the percentage of animals carrying one or more lung tumors. The second is tumor multiplicity, expressed as the average number of tumors per lung per animal, including mice without tumors (Shimkin and Stoner, 1975). Two studies in the literature examined the effects of known carcinogens on strain A/J mouse lung tumors under conditions comparable to the present study, e.g., inhalation exposure for 5 days a week for 6 months (Leong *et al.*, 1971; Adkins *et al.*, 1986). The data are summarized in Table 7. With several known carcinogens, strain A/J mice in group sizes comparable to those in the present experiment responded with both increased tumor incidence and tumor multiplicity. As a general rule, concentrations of the agents calculated as mass were considerably higher than 4 mg/m^3. Naphthalene appeared to be a false negative, since it showed in a later 2-year study some evidence for carcinogenic potential in B6C3F$_1$ mice (National Toxicology Program, 1992). Diesel exhaust gave equivocal results when tested at concentrations of particulate matter similar to the ones used in the present study, and at a concentration of 12 mg/m^3 actually inhibited lung tumor development in strain A mice (Pep-

TABLE 7
Summary of Inhalation Studies with Strain A/J Mice[a]

Agent[b]	Reference[c]	Increased tumor incidence	Increased multiplicity
Bis(Chlormethyl)ether, 1 ppm (4.7 mg/m^3; n = 47)	1	No	Yes
Chloromethyl methyl ether, 2 ppm (6.6 mg/m^3; n = 50)	1	No	ND
Urethan, 138 ppm (502 mg/m^3; n = 50)	1	Yes	Yes
Carbon disulfide, 300 ppm (932 mg/m^3; n = 30)	2	No	No
1,2-Dibromoethane, 20 ppm (153 mg/m^3; n = 60)	2	Yes	Yes
Ethylene oxide, 70 ppm (126 mg/m^3; n = 30)	2	Yes	Yes
Naphthalene, 30 ppm (157 mg/m^3; n = 30)	2	No	No
Vinyl chloride, 50 ppm (128 mg/m^3; n = 30)	2	Yes	Yes
Diesel exhaust 6 mg/m^3	3	No[d]	No[d]
Diesel exhaust 12 mg/m^3	3	No[e]	No[e]
Ozone, 0.5 ppm (1.0 mg/m^3; n = 45)	4	Yes	Yes
NO_2, 10 ppm (18.8 mg/m^3; n = 30)	2	No	Yes
Oxygen, 70%	5	ND	Yes

[a] In all experiments animals were exposed for 6 to 9 months and then terminated for tumor count.
[b] Lowest concentration of agent for eliciting a positive response or highest concentration for yielding negative data. Data in brackets present mg/m^3 with the number of animals in the experimental group.
[c] References: (1) Leong *et al.*, 1971; (2) Adkins *et al.*, 1986; (3) Pepelko and Peirano, 1983; (4) Hassett *et al.*, 1985; (5) Lindenschmidt *et al.*, 1986. ND, no data reported.
[d] One positive study out of two, discounted by authors.
[e] Tumor incidence and multiplicity actually *decreased* in diesel exhaust-exposed A mice.

elko and Peirano, 1983). Surprisingly, ozone and two presumptive noncarcinogens, NO_2 and oxygen, also appeared to have tumorigenic potential in strain A/J mice. Recently, some evidence for ozone carcinogenicity was found in female B6C3F$_1$ mice, but not in male mice (National Toxicology Program, 1993). As far as NO_2 is concerned, it must be mentioned that an earlier study reported that exposure of A/Heston J mice to NO_2 actually decreased tumor multiplicity (Henschler and Ross, 1966). Cell proliferation induced by chronic ozone exposure might explain the development of lung tumors in strain A/J mice exposed to ozone (Witschi, 1991) and hyperoxia might act as a promoting stimulus (Cerutti, 1985). On overall balance, exposure of strain A/J mice for 6 months revealed carcinogenic potential of practically all the known carcinogens tested. A/J mice also appear sensitive to possible tumor-enhancing activity of some other agents.

In this otherwise sensitive test system, SS had no effect. This raises the question of how easily tobacco smoke exposure produces neoplasms in the respiratory tract of mice. In 1975, Shimkin and Stoner concluded that tobacco smoke and its condensates had only weak lung-tumor inducing activity in mice (Shimkin and Stoner, 1975). Later observations seemed to confirm this conclusion. The IARC monograph on tobacco smoking analyzes six chronic exposure studies (IARC, 1986). Disregarding differences in number of cigarettes to which animals were exposed or duration of exposure, the aggregated data show that of an overall total of 1703 mice exposed to tobacco smoke, only 108 animals (6.3%) developed lung tumors. Strains examined were C57B1, BLH, C57B16/Mil, Snell's, and BALB/c. In the corresponding control groups, tumors were found in 39 (3.9%) of 998 animals. Although the lung tumor incidence in the smoke-exposed animals is comparatively small, it nevertheless is almost twice as high as in controls; when tested by χ^2 analysis, the difference is significant. In another study, some 1600 BC3F1/Cum mice were exposed to tobacco smoke for 110 weeks. Lung tumor incidence in the exposed animals was again comparatively low, 5%, and in controls it was 4.3%. While tumor incidence was not significantly higher in the exposed animals, smoke shortened the time to tumor (Henry and Kouri, 1984). In all of these studies, tobacco smoke was a pulmonary carcinogen in mice, although it appeared to have comparatively weak carcinogenic activity.

A few observations are available on lung tumor response to tobacco smoke exposure in the sensitive strain A/J. When kept in a chamber containing reportedly 1 mg per liter (1000 mg/m^3) of tobacco smoke (a concentration that appears to be inordinately high), strain A mice did not respond with increased lung tumor numbers when exposed up to 250 days (Lorenz *et al.*, 1943). The only positive carcinogenic effect of tobacco smoke in strain A mice was reported by Essenberg (1952). He found a 91% tumor incidence in smoke-exposed animals after 1 year, significantly higher than the 59% incidence in controls. One later study confirmed the observation (Essenberg *et al.*, 1955) while another one did not (Essenberg *et al.*, 1956). It was concluded

that nicotine apparently acted as a carcinogen or cocarcinogen (Essenberg, 1957). Unfortunately, no data on chamber concentrations of smoke constituents are available from these studies. This makes it impossible to decide whether in our experiment the failure to observe an effect was because of too short an exposure time, because of too low a chamber concentration of SS, or because SS is not a carcinogen in strain A mice.

An interesting finding were the different K-ras mutation spectra. In spontaneously developing lung adenomas, K-ras mutations occur with approximately equal frequency in codon 12 and codon 61 (You *et al.*, 1989, 1991). Following treatment of strain A mice with methylating or alkylating agents, including the potent lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) or with polycyclic aromatic hydrocarbons, K-ras mutations are predominantly found in codon 12. The pattern is consistent with the known adduct-forming properties of the carcinogens (Belinsky *et al.*, 1989; Ronai *et al.*, 1993; Nesnow *et al.*, 1994). Codon 61 mutations are prominent in lung tumors induced by urethan (Belinsky *et al.*, 1989). It was therefore surprising to find that SS, which contains such well known carcinogens as NNK, benzo[*a*]pyrene, and benz[*a*]anthracene in higher concentration than does mainstream smoke (US EPA 1992), shifted the mutation spectrum from codon 12 toward codon 61. Equally intriguing was the finding that in SS-exposed animals the prominent K-ras mutation was the Arg substitution, a feature that has been considered to be a marker for malignancy in mouse lung tumors (Nuzum *et al.*, 1990). The observations need to be expanded in future studies.

In summary, our paper suggests two conclusions that are not necessarily mutually exclusive. One conclusion is that the strain A mouse lung tumor assay does not detect carcinogenic potential of SS even at concentrations that are in excess of values being called "extreme" when compared to measurements made in the real world (Coggins *et al.*, 1992). The alternative conclusion is that under feasible/practical conditions of exposure, SS is not a lung carcinogen in strain A/J mice.

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